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(54) Title: ANTIGENIC MODULATION OF VIRAL PARTICULES

(57) Abstract

The present invention is directed to a non–immunogenic cellular composition comprising: a cell, particularly a viral particles or virus, having a cell surface and antigenic determinants on the cell surface; an optional linker molecule covalently attached to the cell surface; and a non–immunogenic compounds (e.g., polyethylene glycol or a derivative thereof) covalently attached to the linker molecule or directly to the cell. In one embodiment, the linker molecule is covalently attached directly to the antigenic determinant on the cell surface. In an alternate embodiment, the linker molecule may be covalently attached to a non–antigenic site on the cell surface, but will camouflage the antigenic determinant on the cell surface. Various uses of the resulting non–immunogenic viral cell are also provided, including a method of decreasing phagocytosis of a viral cell, a method of decreasing an adverse reaction to a transfusion, a method of decreasing rejection of a transplanted viral cell, tissue or organ, and a method of decreasing antibody–induced aggregation of cells.

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ANTIGENIC MODULATION OF VIRAL PARTICLES

The present invention relates generally to antigenic modulation of cells, and more particularly to non-immunogenic cellular compositions comprising cells modified with a non-immunogenic compound, and uses of such non-immunogenic cells.

Throughout this application various publications are referenced, many in parenthesis. Full citations for these publications are provided at the end of the Detailed Description.

Acute tissue rejection can be observed in two major clinical situations: 1) blood transfusions; and 2) organ transplantation. In both situations, to be described in greater detail below, antibody binding and complement fixation are the two major mechanisms underlying the destruction of the donor tissue (the donor tissue referring to blood or organs). Previous means of attempting to control acute rejection have centered on tissue matching and pharmacologic interventions. Despite these measures a significant number of often life-threatening acute tissue rejection reactions continue to occur.

Blood transfusions are a crucial component in the treatment of a number of acute and chronic medical problems. These range from massive blood loss following traumatic injury to chronic transfusions to treat diseases such as thalassemia and sickle cell anemia. In most acute injuries simple blood typing (ABO/rh) is sufficient to identify appropriate donors. Occasionally, however, rare blood types are encountered where an appropriate match cannot be quickly found, a situation which may be life-threatening. More often problems are encountered in individuals, usually minorities, receiving chronic transfusions (e.g., as in sickle cell anemia and the thalassemias). Often, simple blood typing becomes insufficient in determining a proper match because these individuals develop transfusion reactions to minor red blood cell antigens. The transfusion reactions to these minor red blood cell antigens can make it nearly impossible to identify appropriate blood donors (Vichinsky et al. 1990).

To date, the only solutions to the above situations are to store autologous blood (frozen or at 4°C), keep a blood bank registry of potential donors with rare blood types, and to encourage minority blood donations. While all of these steps are prudent and variably effective, situations still arise where an appropriate (or even satisfactory) blood match cannot be made. Therefore, a need exists for methods and agents which will disguise otherwise immunogenic (or directly immunologically recognizable) red blood cells.

Similarly, the transplantation of organs (such as kidneys and livers) from one human to another is often made difficult by a lack of exact immunologic identify between donor and recipient. Sometimes, the transplanted organ is subject to direct attack by the immune system of the recipient even before a secondary immunologic response has had time to occur. This so-called 'hyperacute rejection' is often life threatening and, obviously, prevents the effective integration of the transplant into the recipient. Therefore, a need exists for methods and agents which may prevent immediate recognition of the endothelial surfaces of organ transplants, thereby moderating or stopping the process of acute graft rejection. In a similar vein, the transplantation of organs from one species to another ("xenotransplantation") faces even more formidable immunologic barriers and would be greatly facilitated by methods for blocking immunologic recognition of the foreign endothelial surface.

Proteins have been modified by the covalent attachment of soluble polymers such as polyvinyl alcohol, carboxymethyl cellulose (Mitz and Summaria 1961), and polyvinylpyrrolidone (von Spect et al. 1973). Various purified antigenic proteins have also been modified by covalent attachment of polyethylene glycols (PEGs) to render the resulting proteins non-immunogenic. Abuchowski et al. (1977a) disclose the modification of purified bovine serum albumin (BSA) by covalent attachment of methoxypolyethylene glycol, rendering the BSA non-immunogenic. Abuchowski et al. (1977b) disclose the modification of purified bovine liver catalase by covalent attachment of methoxypolyethylene glycol, rendering the catalase non-immunogenic. Jackson et al. (1987) disclose the modification of purified ovalbumin with

monomethoxypolyethylene glycol using cyanuric chloride as a coupling agent. The resulting ovalbumin is non-immunogenic. Various reports have also shown that polyethylene glycol (PEG) coated liposomes have improved circulation time (Klivanov et al. 1991; Senior et al. 1991; Maruyama et al. 1992; and Lasic 1992).

Islets of Langerhans have been microencapsulated in semipermeable membranes in order to decrease immunogenicity of implanted islets (Lacy et al. 1991; Lim 1980). Sawhney et al. (1994) coated rat islets with a polyethylene glycol tetraarylate hydrogel. Importantly, PEG was not directly incorporated into the islet cell membranes but rather the cells were surrounded by the PEG-containing hydrogel.

Zalipsky and Lee (1992) discuss the use of functionalized polyethylene glycols for modification of polypeptides, while Merrill (1992) and Park and Wan Kim (1992) both disclose protein modification with polyethylene oxide.

U.S. Patent No. 4,179,337 of Davis et al. discloses purified polypeptides, such as enzymes and insulin, which are coupled to polyethylene glycol or polypropylene glycol having a molecular weight of 500 to 20,000 daltons to provide a physiologically active non-immunogenic water soluble polypeptide composition. The polyethylene glycol or polypropylene glycol protect the polypeptide from loss of activity and the composition can be injected into the mammalian circulatory system with substantially no immunogenic response.

U.S. Patent No. 5,006,333 of Saifer et al. discloses a biologically persistent, water-soluble, substantially non-immunogenic, substantially non-antigenic conjugate of superoxide dismutase, prepared by coupling purified superoxide dismutase to one to five strands of a polyalkylene glycol which is polyethylene glycol or polyethylene-polypropylene glycol copolymer, wherein the polyalkylene glycol has an average molecular weight of about 35,000-1,000,000.

U.S. Patent No. 5,013,556 of Woodle et al. discloses a liposome composition which contains between 1-20 mole percent of an amphipathic lipid

derivatized with a polyalkylether, as exemplified by phosphatidylethanolamine derivatized with polyethylene glycol.

U.S. Patent No. 5,214,131 of Sano et al. discloses a polyethylene glycol derivative, a purified peptide modified by the polyethylene glycol derivative, and a method for production thereof. The polyethylene glycol derivative is capable of modifying the guanidine groups in peptides. The peptides modified by the polyethylene glycol derivative are extremely stable, are considerably delayed in biological clearance, and retain their physiological activities over a long period.

PCT Application PCT WO 95/06058 published on March 3, 1995 describes the reaction of a sulfonate (sulphonate) ester-activated polymer with a target material. Representative polymers are recited in claims 14-15 and include methoxypolyethylene glycols (MPEG), representative targets are described in claim 16, and target molecules that are parts of cells are disclosed at page 38, lines 28-34. Example 7 shows the reaction of human erythrocytes with activated PMEGF, with covalent bonding of the polyethylene glycol to the erythrocytes, which remain intact.

U.S. Patent No. 5,529,914 describes a method and materials produced by that method which comprises encapsulation (and adherence of) of a biologic material, including a cell, by photopolymerizing an unsaturated macromer, such as a polyethyleneglycol multiacrylate to form a biocompatible membrane (e.g., around the cell). The membrane is described as being immunoprotective (column 6, lines 4-11).

A need continues to exist for methods of making entire cells and tissues and organs, as opposed to purified proteins or peptides, non-immunogenic.

The invention provides a method for modulating the antigenicity and aggregation of mammalian, preferably human, cells. To this end, the subject invention provides for the covalent binding of a non-immunogenic compound to intact cells. Cells that can be effectively modified in accord with the invention include anucleate cells (platelets and red blood cells) and nucleated cells (epithelial cells, endothelial cells, and lymphocytes). In one embodiment, the non-immunogenic compound is polyethylene glycol (PEG) or a derivative

thereof. Potential applications for PEG modification of cells include: 1) PEG-derivatized red blood cells (RBC) to diminish transfusion reactions arising from mismatched blood or sensitization to minor blood group antigens due to chronic transfusions; 2) PEG-derivatization of the vascular endothelium of donor tissues prior to transplantation to prevent/ diminish acute tissue rejection; 3) implantation of PEG-derivatized cells to correct enzyme deficiencies, other inborn errors of metabolism, or other types of defective cellular functions, and 4) transfusion of derivatized RBC into malaria-infected individuals to correct the accompanying acute anemia and prevent the infection of the transfused cells. Unexpectedly, red blood cells modified by PEG have normal *in vitro* and *in vivo* survival when compared to control cells.

Covalent linkage of non-immunogenic compounds (e.g., PEG or PEG-derivatives, such a methoxypolyethylene glycol or PEG-like compounds such as polyethylene oxide), directly or indirectly to membrane proteins of cells, decreases the antigenic recognition of these cells. Some of the available reactions and reagents to accomplish this are summarized in Figure 1. Similarly, insertion of PEG-modified phospholipids/free fatty acids into the cell membrane may serve a similar purpose. The examples hereinbelow demonstrate that unexpectedly (1) it is possible to derivatize normal red blood cells and other cells with PEG without causing lysis, (2) that the derivatized red blood cells remain intact and exhibit normal morphology, (3) that PEG modification of the cell surface does, indeed, 'hide' antigenic determinants such as ABO blood groups, epithelial cell-specific antigens (ESA) and the MHC antigens which underlie tissue/organ rejection, (4) that the derivatized cells survive normally in the circulation of experimental animals, and (5) that PEG derivatized red blood cells from one species have vastly improved survival in the circulation of an animal from another species.

As delineated above, transfusion reactions (to both major and minor red blood cell antigens) represent a significant clinical problem. In most cases, these transfusion reactions actually result from minor surface antigens not routinely measured by blood banks. In situations where either an appropriate blood type

match cannot be located or, more often, when sensitization to minor red blood cell antigens has occurred, PEG-modified red blood cells can be employed to diminish/prevent the recognition of red blood cell antigenic determinants. the application of this invention can also lead to procedures for modification of animal red blood cells which can then be used for transfusion into humans, or into animals of the same or other species. The application of this invention can further lead to procedures for modification of red blood cells to prevent malarial invasion or opsonization by factors such as complement.

In addition, based on the data contained in this disclosure, the scope of this invention extends well beyond blood banking to other areas where foreign tissues are manipulated or introduced *in vitro* or *in vivo*. One area of primary interest is the use of PEG-modified tissues (especially covalent modification of the vascular endothelium) for tissue transplantation. Despite appropriate HLA-matches, many organ transplants fail as a result of immediate tissue rejection. This rejection reaction occurs primarily at the level of the vascular endothelium and results in vessel occlusion, tissue hypoxia/ischemia and ultimate loss of the organ transplant. Based on the chemistry of PEG-cell derivatization disclosed herein, it is possible to perfuse the vasculature of the tissue with a solution of activated PEG. This will modify the vessel walls (i.e., endothelial cells) which will prevent or diminish the aforementioned immediate tissue rejection. This technology can thus improve the rate of successful tissue engraftment.

The invention thus provides a non-immunogenic cellular composition comprising: a cell having a cell surface and antigenic determinants on the cell surface; and a non-immunogenic compound covalently attached to the cell surface directly or by means of the linking moiety, which linking moiety can be derived from a linker molecule, as discussed below. The non-immunogenic compound acts to block recognition of the antigenic determinants on the cell surface. In one embodiment, the linking moiety is covalently attached directly to the antigenic determinant on the cell surface. In an alternate embodiment, the linking moiety may be covalently attached to a non-antigenic site of the cell

surface, the antigenic site on the cell surface is camouflaged or masked by virtue of the long chain length of the non-immunogenic compound.

The invention further provides a method of producing a non-immunogenic cell. The method comprises: covalently attaching a non-immunogenic compound to the surface of the cell directly, or by means of a linking moiety, so that non-immunogenic compound blocks recognition of antigenic determinants on the cell surface to produce a non-immunogenic cell. A non-immunogenic cell produced by this method is also provided by the subject invention.

The concept of the subject invention can also provide a method of decreasing phagocytosis of a cell. This method comprises: selecting a cell for introduction into a subject, the cell having a cell surface and antigenic determinants on the cell surface; covalently attaching an amount of a non-immunogenic compound to the cell surface directly or by means of a linking moiety, so that the attached non-immunogenic compound blocks recognition of antigenic determinants on the cell surface to produce a non-immunogenic cell; and introducing the non-immunogenic cells into a subject, wherein phagocytosis of the non-immunogenic cell is decreased as compared to phagocytosis of the cell prior to modification.

Further provided is a method of decreasing an adverse reaction to a transfusion, the method comprising: selecting a red blood cell for transfusion into a subject, the red blood cell having cell surface and blood group antigenic determinants on the cell surface; covalently attaching a non-immunogenic compound capable of blocking the blood group antigenic determinants on the cell surface, to the cell surface directly or by means of a linking moiety, so as to produce a non-immunogenic red blood cell; and transfusing a subject with the non-immunogenic red blood cell, wherein adverse reaction to the transfusion of the non-immunogenic red blood cell is decreased as compared to transfusion of the red blood cell prior to modification.

Also provided is a method of decreasing rejection of a transplanted cell, the method comprising: selecting a cell for transplantation into a subject, the cell

having a cell surface and antigenic determinants on the cell surface; covalently attaching a non-immunogenic compound capable of blocking the recognition of the antigenic determinants on the cell surface, to the cell surface directly or by means of a linking moiety, so as to produce a non-immunogenic cell; and transplanting the non-immunogenic cell into a subject, wherein rejection of the transplanted cell is decreased as compared to rejection of the cell prior to modification.

The invention provides a method of decreasing aggregation of nucleated and anucleate cells such as that induced by antibodies or by other cell:cell interactions. The method comprises: covalently attaching non-immunogenic compounds capable of blocking recognition of antigenic determinants on a cell surface to the cell surface of each of a plurality of cells directly or by means of a linking moiety, so as to produce non-aggregating cells, wherein antibody-induced aggregation of the non-aggregating cells is decreased as compared to antibody-induced aggregation of the cells prior to modification.

As used herein, the term "linking moiety" or "linker" refers to an at least divalent organic group that covalently, or by complexation or chelation binds to both the non-immunogenic molecule and the cell surface, to attach at least one non-immunogenic compound to at least one functional group or structure on the cell surface. The linking moieties can be derived from reactive linker molecules, as described hereinbelow.

These and other features and advantages of this invention will be evident from the following description of preferred embodiment when read in conjunction with the accompanying drawings in which:

Fig. 1 is a schematic depiction of the preparation of certain embodiments of the non-immunogenic cellular compositions according to the subject invention;

Fig. 2 is a schematic depiction of a further embodiment of a non-immunogenic cellular composition according to the subject invention. In this embodiment, the non-immunogenic compound is polyethylene glycol or a derivative thereof and the activated PEG (PEG-linker) is covalently attached to

antigenic determinants on the cell surface (directly blocking antigenic sites) and also covalently attached to non-antigenic sites on the cell surface (indirectly blocking antigenic sites due to their long chain length);

Fig. 3 is a graph showing that monomethoxypoly(ethylene glycol) (mPEG) modification of red blood cells causes a dose-dependent inhibition of anti-A antibody induced RBC aggregation defined turbidometrically;

Fig. 4 is a bar graph showing that mPEG modification of red blood cells only slightly increases red blood cell lysis;

Fig. 5 is a graph showing the mPEG modification of red blood cells has no effect on red blood cell osmotic fragility;

Fig. 6 is a bar graph showing that mPEG-modified type A red blood cells bind significantly less anti-A antibody;

Fig. 7 is a bar graph showing that mPEG-modified sheep red blood cells are significantly less prone to phagocytosis by human peripheral blood monocytes;

Fig. 8 is a graph showing no significant differences in the *in vivo* survival of control mouse red blood cells and mouse red blood cells modified with activated PEG; and

Fig. 9 is a graph demonstrating that sheep red blood cells (solid symbols) enter and survive within the circulatory system of a mouse whereas unmodified sheep red blood cells (open symbols) do not.

Fig. 10 is a graph depicting the Donor A peripheral blood mononuclear cell (PBMC) response to antigenically foreign Donor B PBMC (Panel A) and the Donor B PBMC response to Donor A (Panel B) in MLC analysis of control and mPEG derivatized PBMC.

Fig. 11 is a graph demonstrating that platelet aggregation is prevented by the covalent modification of platelet surfaces with mPEG.

Fig. 12 is a bar graph demonstrating that mPEG-modification of epithelial cells blocks antibody recognition of surface antigens.

Figure 13 is a blot demonstrating that Simian Vacuolating virus 40 (SV40) shows a shift in coat protein electrophoresis.

The present invention provides a non-immunogenic cellular composition comprising: a cell having a cell surface and antigenic determinants on the cell surface; a linking moiety covalently attached to the cell surface; and a non-immunogenic compound covalently attached to the linking moiety and capable of blocking recognition of the antigenic determinants on the cell surface. Alternatively, the non-immunogenic compound can be bound directly to the cell surface, if it comprises groups such as carboxylic acids, aldehydes, ketals or acetals that are reactive with NH_2 or SH groups on the cell surface.

The present invention is differentiated from the invention of copending U.S. Patent Application 08/671,452, identified above, in that the present invention is specifically directed towards another class of cell which can be readily modified by the same materials used on red blood cells, lymphocytes, etc., that class of cell being viruses (more appropriately "virus particle"). As demonstrated in Figure 13, Simian Vacuolating virus 40 (SV40) shows a dramatic shift in coat protein electrophoresis following viral modification with mPEG. This modification may be useful for at least two reasons: 1) high degrees of derivatization may result in apparent viral inactivation; and 2) varying degrees of derivatization may be useful in preventing immune recognition of viral vectors (e.g., adenovirus) currently used as gene vectors in gene therapy. Interestingly, the change in viral inactivation may result from either actual particle inactivation, or by preventing viral invasion of the cell (defect in cell:cell interaction). In contrast to viral inactivation, low levels of derivatization may impede immunologic recognition while only modestly affecting viral infectivity. In certain circumstances this decreased antigenicity may be useful. A case-in-point is the use of adenovirus as a vector in gene therapy. Primary inoculation (via inhalation) of adenovirus in cystic fibrosis patients have demonstrated effectiveness in ameliorating disease severity. However, subsequent administrations of the adenovirus vector are progressively less effective due to initiation of an effective immune response against the viral coat proteins by the

patient. Thus, by varying the degree of viral derivatization, a balance can be achieved between viral inactivation (i.e., impaired cell invasion) and decreased immune recognition of the viral coat proteins by the patients immune system. Sufficiently coated viruses may avoid detection/inactivation by the immune system while still giving rise to productive therapeutic invasion of the lung epithelial cells.

mPEG modification of the external aspect of the cell membranes effectively "hides" major and minor antigenic determinants on a large variety of cell types. Furthermore, viral particles can also be readily derivatized. This camouflage occurs as a consequence of both decreased antibody binding and alteration in cell:cell interaction. These findings are supported by the (1) lack of gross antibody-induced agglutination, (2) significantly decreased antibody binding, (3) diminished phagocytosis by heterologous macrophages, (4) loss of T cell proliferation in the mixed lymphocyte reaction, and (5) loss of platelet aggregation up derivatization. Importantly, treated cells remain structurally and metabolically normal. This is readily demonstrated by derivatized RBC which exhibit normal morphology, deformability, osmotic fragility, oxygen binding during *in vitro* incubations. The "normal" nature of the modified RBC is further demonstrated by normal *in vivo* survival of mPEG-mouse RBC.

Our research leads us to believe that the covalent attachment of nonimmunogenic materials to intact cells (e.g., RBC, endothelial cells, epithelial cells, pancreatic β cells, viruses *etc.*) may have significant clinical implications. These include, but are not limited to: 1) derivatized RBC to diminish transfusion reactions arising from mismatched blood or sensitization to minor blood group antigens due to chronic transfusions; 2) derivatization of the vascular endothelium of donor tissues prior to transplantation to prevent/diminish acute tissue rejection; 3) implantation of derivatized cells to correct enzyme deficiencies; 4) transfusion of derivatized RBC into malaria-infected individuals to correct the accompanying acute anemia and prevent infection of the transfused cells; (5) viral infectivity (e.g., SV40, Adenovirus, HIV) may be dramatically decreased (due to altered cell:cell interaction) by modification of the viral coat

proteins; (6) use of mPEG (or other nonimmunogenic tails) may host prevent immune recognition and improve virus-mediated gene therapy (e.g., as currently being investigated in the treatment of cystic fibrosis); and (7) variations of the derivatization procedure may be useful in impeding cancer metastases via disruption of cell:cell interaction and subsequent prevention of tissue invasion.

The cell can be any suitable cell with accessible antigenic determinants on the cell's surface. Suitable cells include anuclear cells, for example, hematopoietic cells, i.e., red blood cells or platelets, or nucleated cells, for example, vascular endothelial cells, PBMCs, hepatic cells, neuronal cells, pancreatic cells, or epithelial cells.

The antigenic determinants on the cell surface can be due to the presence of antigenic proteins, antigenic carbohydrates, antigenic sugars, antigenic lipids, antigenic glycolipids, antigenic glycoproteins, etc. "Antigenic" determinants can also be involved in malarial invasion of a cell, or opsonization of a cell. For example, red blood cells have antigens on their surface which determine ABO/rh blood types. These antigens are often referred to as blood group antigenic determinants. These antigens are recognized by an incompatible host and the donor cell will be rapidly destroyed. This can involve the enhancement of natural immunity (through phagocytes, such as macrophages, neutrophils, and natural killer cells) or the stimulation of specific or acquired immunity (including humoral immunity through antibodies and cell-mediated immunity through T lymphocytes). In any event, the cell is recognized as foreign and elicits an immune response.

To prevent this immune response from destroying the cell, the subject invention involves modification of the antigenicity of the cell. This modification is accomplished by attaching a non-immunogenic compound to the cell. Suitable non-immunogenic compounds for use in the subject invention are non-immunogenic compounds capable of blocking recognition of antigenic determinants on the cell surface. The compounds are generally long chain compounds, wherein the long chain can sterically block the antigenic determinants. Such non-immunogenic compounds include polyalkylene glycols

such as polyethylene glycol, polypropylene glycol, mixed polypropylene-polyethylene glycols, or derivatives thereof (including monomethoxypolyethylene glycol), certain polysaccharides such as dextrans, cellulosics, Ficoll, and arabinogalactan, as well as synthetic polymers such as polyurethanes. Useful molecular weights of these compounds can range from about 100-500 to 100,000-200,000 Daltons or above.

The presently preferred non-immunogenic compound according to the subject invention is polyethylene glycol or a derivative thereof. The polyethylene glycol or derivative thereof is a molecule with a very long chain length. The non-immunogenic compound (e.g., polyethylene glycol or derivative thereof) can be directly attached to an antigenic site (e.g., an antigenic determinant) on a cell surface via a linking moiety (direct modification of antigenicity) (see Fig. 1 and Fig. 2) or can be attached to a non-antigenic site on the cell surface via a linking moiety. In both cases, the long chain of the non-immunogenic compound (e.g., polyethylene glycol or derivative thereof) effectively blocks antigenic sites on the cell surface (indirect modification of antigenicity) (see Fig. 2). In either embodiment, the non-immunogenic compound (e.g., polyethylene glycol or derivative thereof) is attached to the cell surface by a linking moiety, which is derived from a linker molecule that can react with the PEG. The combination of a polyethylene glycol or derivative thereof and the linker molecule is generally referred to as an "activated" polyethylene glycol or derivative thereof.

Polyethylene glycols (PEG) and derivatives thereof are well known in the art. Polyethylene glycol has the formula

$$H(OCH_2CH_2)_nOH$$

wherein n is greater than or equal to 4, with a molecular weight of up to about 20,000 Daltons. However, PEGs and derivatives thereof are available having molecular weights of 200,000 Daltons and above, and can be used in the practice of the present invention, alone, or in combination with lower m.w. materials.

Various derivatives of polyethylene glycol comprise substitutes for the H or OH end groups, forming, for example, polyethylene glycol ethers (such as

PEG-O-R; PEG-O-CH_3; CH_3-PEG-OH or "mPEG"; 2,4-dinitrophenyl ethers of PEG), polyethylene glycol esters (such as PEG-$O_2C(CH_2)_{14}CH_3$; PEG-$O_2CCH_2CH_2CO_2$-atropine), polyethylene glycol amides (such as PEG-$O_2C(CH_2)_7CONHR$; mPEG-$O_2CCH_2CH_2CONH(CH_3)CHCH_2C_6H_5$; PEG-$O_2CCH_2CH_2CONHCH_2CH_2$-$NAD^+$), polyethylene glycol amines (such as PEG-NH_2; PEG-$NH(CH_2)_6NH_2$; PEG-$OCH_2CH_2NH_2$; mPEG-NH_2), polyethylene glycol acids (such as PEG-$O_2C(CH_2)_2CO_2H$; PEG-OCH_2CO_2H; PEG-$O_2C(CH_2)_7$-CO_2H), polyethylene glycol aldehydes (PEG-O-CH_2-CHO), and electrophilic derivatives (such as PEG-Br; PET-OSO_2CH_3; PEG-OTs). Various phenyl moieties can also be substituted for the H or OH of PEG, such as the 2,4-dinitrophenyl ether of PEG mentioned above).

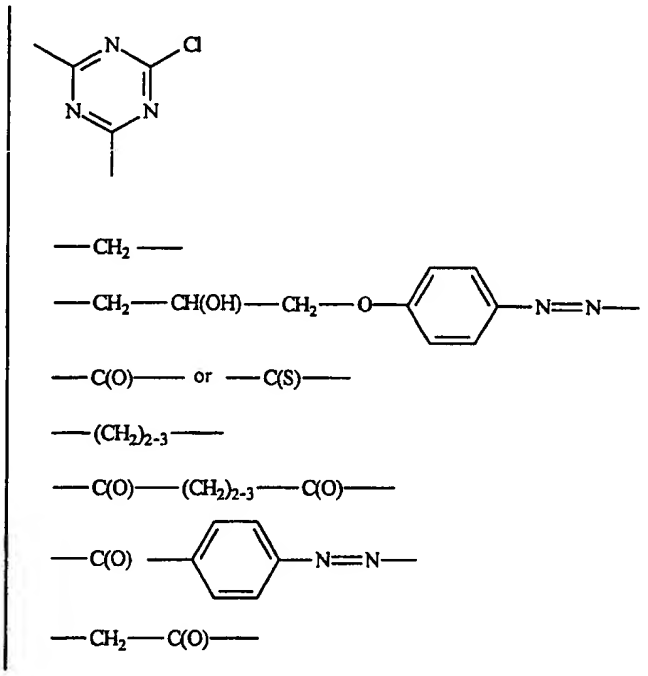
For a full discussion of polyethylene glycol and activated derivatives thereof, including the synthesis of the derivatives, see the following references: Harris et al. 1984; Harris 1985; Zalipsky and Lee 1992; Park and Kim 1992; Merrill 1992; and U.S. Patent Nos. 4,179,337 and 5,214,131. The particular non-immunogenic compounds, including the polyethylene glycol derivatives, listed above are exemplary only, and the invention is not intended to be limited to those particular examples.

According to the subject invention, these non-immunogenic compounds (e.g., polyethylene glycol molecules or derivatives thereof) are covalently attached to the cell surface by means of a linking moiety. These linking moieties can be prepared by reaction of the polyethylene glycol or derivative thereof with suitable linker molecules that are also well known in the art, and include, for example, cyanuric chloride, imidazolyl formate, succinimidyl succinate, succinimidyl glutarate, N-hydroxysuccinimide, 4-nitrophenol, and 2,4,5-trichlorophenol. These linker molecules 'activate" the PEG, a term also well known in the art. For a description of activation of PEG, with examples of known linking moieties and molecules, see Harris 1985. The linker molecules listed above are exemplary only, and the invention is not intended to be limited to those particular examples. As would be recognized by one of skill in the art, the linking molecules disclosed hereinabove and on Figure 1 react with a

reactive group such as a hydroxy of the non-immunogenic compound, e.g., the PEG or MPEG, and also react with an NH_2 or, in some cases, SH, group of a peptidyl or other amino acid residue on the cell surface to covalently join them, whereby the linking molecule is converted in one or more steps into a divalent linking moiety such as shown on Table 1, below.

Table 1

Linking Moiety

[Non-immunogenic compound]-O-  -NH-Cell

A number of "activated" methoxypolyethylene glycols are commercially available, in which mPEG (m.w. 5000) has been bound to a linking molecule at the hydroxyl terminus. These include, methoxypolyethylene glycol (mPEG) para-nitrophenyl carbonate, mPEG cyanuric chloride, mPEG-succinimidyl succinate, mPEG tresylate, and mPEG imidazolyl carbonyl. For example, see I. Jackson et al., Anal. Biochem., 565, 114 (1987); A. Abuchowski et al., J. Biol. Chem., 252, 3578 (1977); F. M. Veronese et al., Appl. Biochem. Biotech., 11, 141 (1985), C. Delgado et al., Biotech. Appl. Biochem., 12, 119 (1990); C. O. Veavchemp et al., Anal. Biochem., 131, 25 (1983).

The chemistry involved in the covalent attachment of the non-immunogenic compound (such as PEG or a derivative thereof) to reactive groups such as proteins and peptides on the cell surface (thus, covalent attachment of the

non-immunogenic compound to a cell surface) by means of linking moieties, is known in the art, and is discussed in detail in Harris 1985; Harris et al. 1984; and Zalipsky and Lee 1992. Because polyethylene glycol and its derivatives are very well known in the art, including the synthesis and modification thereof, including attachment to proteins, further details are not disclosed herein relating to this aspect of the invention, other than the examples that follow.

Having thus identified the non-immunogenic cellular composition according to the subject invention, various uses of the invention are possible.

The invention thus further provides a method of producing a non-immunogenic cell. The method comprises: covalently attaching a non-immunogenic compound capable of blocking recognition of antigenic determinants on a cell surface, to a cell surface, directly, or by means of a linking moiety, so as to produce a non-immunogenic cell. If the cell is a red blood cell, the method can further comprise transfusing a subject with the non-immunogenic cell. Since the antigenic determinants, such as the blood group antigenic determinants, on the red blood cell are blocked by the non-immunogenic compound, the transfused non-immunogenic red blood cell will not elicit an immune response. As discussed above, this method can be very useful when red blood cells need to be transfused quickly without the availability of complete blood typing or cross-matching, or when unmatched from a subject is available.

If the cell is part of a tissue or organ, the method can further comprise transplanting the non-immunogenic tissue or organ into a subject. Since the antigenic determinants on the tissue or organ, such as the vascular endothelial cells which form an exposed antigenic surface of the tissue or organ, are blocked by the non-immunogenic compound, the transplanted non-immunogenic tissue or organ will not elicit an immune response. As discussed above, this method is very useful to avoid severe rejection reactions, or graft vs. host disease, when organs or tissues are transplanted.

The invention further provides a non-immunogenic cell produced by the above method.

The concept of the subject invention can also provide a method of decreasing phagocytosis of a cell. This method comprises: introducing the non-immunogenic cell into a subject, wherein phagocytosis of the non-immunogenic cell is decreased as compared to phagocytosis of the cell prior to modification. The non-immunogenic cell can be prepared by a process comprising: selecting a cell for introduction into a subject, the cell having a cell surface and antigenic determinants on the cell surface; covalently attaching to the cell surface, directly or by means of a linking moiety, a non-immunogenic compound that blocks recognition of the antigenic determinants on the cell surface, so as to produce a non-immunogenic cell. In the case where the cell is a red blood cell, this method can prevent phagocytosis of the "foreign" red blood cell, by rendering the red blood cell non-immunogenic. The "foreign" red blood cell may be from another human, or may be from another non-human subject. In either case, the body's response would be to attempt to eliminate the "foreign" red blood cell including by phagocytosis.

Further provided is a method of decreasing an adverse reaction to a transfusion, the method comprising: transfusing a subject with the non-immunogenic red blood cell, wherein adverse reaction to the transfusion of the non-immunogenic red blood cell is decreased as compared to transfusion of the red blood cell prior to modification. The non-immunogenic red blood cells are prepared by selecting a red blood cell for transfusion into a subject, the red blood cell having a cell surface and blood group antigenic determinants on the cell surface; covalently attaching to the cell surface a non-immunogenic compound in an amount capable of blocking the blood group antigenic determinants on the cell surface; wherein the compound is covalently attached to the cell surface directly or by means of a linking moiety, so as to produce a non-immunogenic red blood cell. As discussed above, the red blood cell could be from another human or from a non-human mammal.

Also provided is a method of deceasing rejection of a transplanted cell, the method comprising: transplanting a non-immunogenic modified cell into a subject, wherein rejection of the transplanted modified cell is decreased as

compared to rejection of the cell prior to modification. The cell is prepared by a process comprising: selecting a cell for transplantation into a subject, the cell having a cell surface and antigenic determinants on the cell surface; covalently attaching a non-immunogenic compound to the cell surface directly or by means of a linking moiety, so that the non-immunogenic compound blocks the recognition of the antigenic determinants on the cell surface, to produce a non-immunogenic cell. Where the cell is part of a tissue or organ which is to be transplanted into a subject, a preferred method of carrying out the covalent attachment is to perfuse the tissue or organ with a solution of an activated polyethylene glycol or derivative thereof (i.e., the polyethylene glycol or derivative thereof is first attached to the linker molecule, forming an activated PEG, which is then perfused over the tissue or organ). During the perfusion, the activated PEG covalently attaches to the cell surface via a linking moiety.

The invention provides a method of decreasing antibody-induced aggregation of cells, the method comprising: covalently attaching to the cell surface non-immunogenic compounds capable of blocking recognition of antigenic determinants on the cell surface; wherein the compounds are covalently attached to the cell surface of each of a plurality of cells, directly or by linking moieties, so as to produce non-aggregating cells, wherein antibody-induced aggregation of the non-aggregating cells is decreased as compared to antibody-induced aggregation of the cells prior to attachment of the compounds. This method is particularly applicable where the cells are red blood cells, and where the antigenic determinants on the cell surface comprise blood group antigenic determinants.

In each of the above-described methods, a linker molecule can be first reacted with the non-immunogenic compound (forming an "activated" compound) and then the linker molecule can be reacted with the cell surface. The order of these steps can be reversed, and any reference to the two steps is intended to cover the two steps in either order. Accordingly, the linker molecule can also be attached to the cell surface, then the non-immunogenic compound

can be reacted with the linker molecule to bind it to the cell surface via a thus-formed linking moiety, in accordance with the claims and disclosure herein.

In the examples which follow, PEG modification of the external aspect of the red blood cell membrane effectively 'hides' major antigenic determinants such as ABO blood group substances. This is evident in the (1) lack of gross antibody-induced agglutination, (2) significantly decreased antibody-induced aggregation, and (3) diminished phagocytosis by heterologous macrophages. Treated red blood cells remain intact, exhibiting only minor spontaneous hemolysis, and demonstrate normal osmotic fragility over at least 48 hours *in vitro* incubation. The "normal" nature of the modified mouse red blood cell is further demonstrated by normal *in vivo* survival.

The PEG modification procedure is surprisingly well tolerated by the cells, yielding a product which survives normally in the circulation. The derivatized cells are antigenically disguised and not recognized by blood group antibodies or by phagocytes. Perhaps most surprisingly, treated red blood cells from one species survive much longer than do untreated red blood cells in the circulation of another species.

The invention thus provides for (1) derivatization of human red blood cells to permit transfusions into people difficult to match (because they have pre-existing antibodies to minor blood groups); (2) derivatization of human red blood cells to permit transfusions into people of unknown blood groups who may even differ in major (e.g., ABO) blood groups from the donor; (3) derivatization - by perfusion of activated mPEG solutions - of human organ grafts to prevent unexpected hyperacute rejection episodes; (4) derivatization - by perfusion of activated mPEG solutions - of organs from non-human animals to prevent hyperacute rejection and to improve the chances of ultimate successful engraftment in humans.

EXAMPLE I

Inhibition of Red Bl od Cell Agglutination:

Normal red blood cells (erythrocytes) were washed 3 x in isotonic saline. A red blood cell suspension of hematocrit about 12% is prepared in isotonic alkaline phosphate buffer (PBS; 50 mM K_2HPO4 and 105 mM NaCl, pH about 9.2). Cyanuric chloride-activated methoxypolyethylene glycol (Sigma Chemical Co.) is added and the red cells are incubated for 30 minutes at 4°C. Cell derivatization can also be done under other pH and temperature conditions with comparable results to those presented. For example, red blood cells derivatized at pH 8.0 for 60 minutes at 22°C demonstrated virtually identical characteristics to those derivatized at pH 9.2 for 30 minutes at 4°C. The extreme range of pH and temperature conditions make this procedure broadly applicable to a wide range of cells and tissues. The proposed mechanism of covalent reaction with external proteins and other membrane components is outlined below. Typical activated mPEG concentrations used range from 0 to 8 mg per ml of red blood cell suspension. The typical activated mPEG concentration to be used on other anuclear (i.e., platelets) and various nucleated cells (e.g., vascular endothelial, hepatic, hematopoietic, neuronal, pancreatic cells, epithelial cells, etc.) can readily be determined in view of the teachings herein.

As shown in Figure 3, the covalent binding of mPEG to the membrane proteins of intact red blood cells prevents red blood cell agglutination. This is apparent at the gross level using agglutination induced by ABO antibodies, and at a finer level using a platelet aggregometer modified to measure red blood cell aggregation (Fig. 3). Type A red blood cells were treated with 0, 3, or 6 mg cyanuric chloride-activated mPEG (m.w. 5000) per ml of blood and incubated at 4°C for 30 minutes. The cells were washed 3 times with isotonic saline and resuspended to a 40% hematocrit in saline.

For gross agglutination, equal volume of a RBC suspension of hematocrit 40% and a commercially available anti-A blood typing antibody (Carolina Biological Supply) were mixed and photographed. Increasing amounts of bound mPEG effectively inhibited the agglutination reaction. In the absence of

derivatization, a typical blood typing response was observed. In contrast, with increasing amounts of covalently bound mPEG, a dose-dependent decrease in sera-induced agglutination of RBC was observed. Indeed, at 6 mg mPEG/ml RBC, no detectable agglutination was observed at the gross level.

Fig. 3 shows red blood cell microaggregation as measured at 37°C in a platelet aggregometer. As shown, mPEG modification caused a dose-dependent inhibition of anti-A antibody induced red blood cell aggregation.

Further testing of matched control and mPEG-derivatized RBC selected minor RBC antigens also demonstrated a significant decrease in the antigenicity of the mPEG-modified RBC (Table 2).

Table 2

Detection of Selected Rh and MNS PBS Antigens on Control and Derivatized RBC

Antigen	C	c	E	e	K	S	s
Control	0	4^+	0	3^+	0	3^+	3^+
mPEG-Treated	0	1^{+w}	0	1^{+w}	0	1^+	1^{+w}

Agglutination response is measured macroscopically with a $4+^s$ rating being the strongest and $1+^w$ being the weakest agglutination response. As shown, in all cases where a minor RBC antigen was detected, mPEG-modification virtually abolished its detection (e.g., 4^+ to 1^{+w}). Importantly, the degree of activated mPEG derivatization used in this study was relatively low (6 mg/ml) in comparison to the levels which can be used (up to approximately 30 mg mPEG/ml RBC) while exhibiting no adverse effects on the RBC. Indeed, based on the mPEG-dose dependency noted in Fig. 3, it is very likely that higher degrees of derivatization will likely further suppress antigen detection.

EXAMPLE II

Effect on Red Blood Cell Stability:

While mPEG-modification of red blood cells slightly increases red blood cell lysis, this lysis is less than 5% of the total red blood cell mass (Fig. 4). Furthermore, mPEG-attachment was found to have no effect on red blood cell osmotic fragility (Fig. 5). Red blood cell stability was minimally modified by

the covalent attachment of mPEG. As shown in Fig. 4, red blood cell lysis was slightly increased by the attachment of mPEG. However, red blood cell lysis of the RBC during mPEG modification followed by 24 hours storage at 4°C or after incubation at 37°C was less than 5%. As shown in Fig. 5, osmotic fragility of the mPEG-treated red blood cells was also unaffected. Shown are the osmotic fragility profiles of control and mPEG-modified (3 and 6 mg/ml) red blood cells after 48 hours incubation at 37°C. Again, while a very minor increase in spontaneous lysis was observed, no significance differences in the osmotic lysis profiles were seen. Electron micrographic analysis of control and mPEG-derivatized RBC also demonstrate no apparent structural changes.

EXAMPLE III

Inhibition of Antibody Binding:

mPEG-modified red blood cells bind significantly less anti-A antibody (Fig. 6). As shown in Fig. 6, an ELISA assay of mPEG-treated human blood type A^- red blood cells demonstrates significantly less antibody binding by mPEG-modified red blood cells. The control and mPEG red blood cells were mixed with an IgG anti-A antibody incubated for 30 minutes. The samples were extensively washed and a secondary antibody (anti-human IgG conjugated with alkaline phosphatase) was added to quantitate bound anti-Blood group A antibody.

EXAMPLE IV

Inhibition of Phagocytosis of Foreign Cells:

mPEG-modified sheep red blood cells are significantly less prone to phagocytosis by human peripheral blood monocytes (Fig. 7). As would be indicated by decreased antibody binding (Fig. 6), mPEG-modified sheep red blood cells are significantly less susceptible to IgG-mediated phagocytosis by human peripheral blood monocytes. mPEG-modified sheep red blood cells were incubated with human peripheral blood monocytic cells for 30 minutes. The uningested red blood cells were removed by hypotonic lysis and the number of monocytes containing sheep red blood cells, as well as the number of sheep red blood cells ingested, were determined microscopically.

EXAMPLE V

mPEG-Derivatized Mouse Red Blood Cells Have Normal *In Vivo* Survival:

As shown in Fig. 8, no significant differences were noted in the *in vivo* survival of control red blood cells and red blood cells modified with either 3 or 6 mg/ml activated mPEG. *In vivo* survival of control and mPEG-modified mouse red blood cells was determined using a fluorescent fatty acid label (PKH-26; Sigma Chemical Company). Blood was obtained from donor BALB/C mice, treated with 0, 3, or 6 mg/ml activated mPEG and washed thrice. The washed cells were then labeled with PKH-26 and injected *i.p.* into naive BALB/C mice. Blood samples were obtained by tail-cuts at the indicated time points and analyzed via FACScan.

EXAMPLE VI

mPEG-Derivatization of Sheep Red Blood Cells Results in Enhanced *In Vivo* Survival in Mice:

Comparable numbers of mPEG-modified sheep red blood cells (mPEG-sRBC) were injected *i.p.* into BALB/C mice. As shown in Fig. 9, mPEG-sRBC showed a greater rate of entry into the peripheral circulation and demonstrated longer *in vivo* survival in mice. *In vivo* survival of mPEG-sRBC in mice was determined using a fluorescent fatty acid label (PKH-26; Sigma Chemical Company). Blood was obtained from a donor sheep and treated with 0 or 6 mg/ml activated mPEG and washed thrice. The washed sheep red blood cells were labeled with PKH-26 and injected *i.p.* into naive BALB/C mice. Blood samples were obtained by tail-cuts at the indicated time points and analyzed via FACScan.

EXAMPLE VII

mPEG-Modulated Lymphocytes:

The mixed lymphocyte culture (MLC) is a very sensitive measure of histocompatibility between donor and recipient. Indeed, though time consuming, this assay is perhaps the best indicator of the probability of tissue transplant survival in the organ recipient. Primarily the MLC measures the antigenic variance between the HLA complex (the primary antigens responsible

for tissue compatibility in transplants) between two individuals. As shown in Figure 10, covalent modification with mPEG of lymphocytes from either donor results in a virtually complete inhibition of recognition of the antigenically foreign lymphocytes. Shown is the proliferation, measured by ^{3}H-thymidine incorporation into DNA, of responder cells in response to a fixed concentration (2.5×10^{5} PBMC) of stimulator (i.e., cells irradiated to prevent cell replication). Panel A demonstrates PBMC Donor A's response to antigenically foreign Donor B PBMC. Panel B demonstrates Donor B's response to Donor A. In contrast, the population of responder (i.e., nonirradiated) cell expands tremendously in response to control irradiated PBMC (peripheral blood mononuclear cells).

These results are further confirmed by photomicrographs of the mixed lymphocyte cultures. Extensive proliferation, cell spreading, and expansive foci of responder cells are seen in response to control stimulator cells. In contrast, the same population of responder cells fails to recognize mPEG-treated stimulator cells, remain morphologically unactivated and fail to proliferate.

EXAMPLE VIII

Modification of Platelets:

Other blood cells are also amenable to mPEG modification. Platelets were modified at pH 8.0 for 60 minutes at room temperature by the procedure of Example 1. The dotted line represents platelet rich plasma (PRP) in the absence of ADP (i.e., control unactivated platelets). As demonstrated in Figure 12, mPEG derivatized platelets do not aggregate in response to activation by ADP (5 μM). While control platelets are fully aggregated within approximately 2 minutes, mPEG-modified platelets remain unaggregated even after 7 minutes of exposure to ADP. The loss of aggregation is mediated by disruption of cell:cell interaction (i.e., preventing platelet interaction and microaggregate formation). Indeed, alteration in cell:cell interaction is a primary event due to the covalent modification of cell surfaces with non-immunogenic materials.

EXAMPLE IX

Modification of Epithelial Cells:

To determine if non-hematological cells could be antigenically modified by mPEG-derivatization, a breast carcinoma epithelial cell line (MCF7) was examined. A mouse monoclonal antibody directed towards epithelial specific antigen (ESA; a 40 kD glycoprotein) was chosen. Mouse anti-human ESA binding was quantitated using a BD-FACScan. FITC-conjugated goat anti-mouse antibody was used to detect bound ESA. Epithelial cell concentration was 5 x 10^5 cells/ml with a 1:6000 titre of anit-ESA antibody. Epithelial cells were derivatized using a modification of the RBC-derivatization protocol. Specifically, confluent monolayers of MCF7 cells were scraped from tissue culture flasks and suspended in RPMI media. The cell suspensions were incubated with increasing concentrations of activated mPEG at pH 8.0 and incubated at room temperature for 60 minutes. The cells were then washed 3 x with culture media prior to the antibody binding assay.

As shown in Figure 12, a mPEG-dose dependent decrease in ESA-specific antibody binding was observed. At the highest mPEG dosage used (8 mg/ml cells) a > 70% decrease in anti-ESA binding was observed.

The covalent modification of the external cell membrane with non-immunogenic materials (e.g., mPEG) effectively "hides" both major and minor antigenic determinants on a large variety of nucleated and anucleated cells. The covalent attachment of non-immunogenic materials to intact cells (e.g., RBC, endothelial cells, epithelial cells, pancreatic β cells, etc.) can be used for: (1) Derivatization of human red cells to permit transfusions into people difficult to match (because they have pre-existing antibodies to minor blood groups); (2) Derivatization of human red cells to permit transfusions into people of unknown blood groups who may even differ in major (e.g., ABO) blood groups from the donor; (3) Derivatization, by perfusion of mPEG solutions, of human organ grafts to prevent unexpected hyperacute rejection episodes; (4) Derivatization - by perfusion of mPEG solutions - of organs from non-human

animals to prevent hyperacute rejection and to improve the chances of ultimate successful engraftment.

Although preferred embodiments have been depicted and described in detail herein, it will be apparent to those skilled in the relevant art that various modifications, additions, substitutions and the like can be made without departing from the spirit of the invention and these are therefore considered to be within the scope of the invention as defined in the claims which follow.

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WHAT IS CLAIMED IS:

1. A non-immunogenic cellular composition comprising:

a viral cell having a cell surface and antigenic determinants on said cell surface;

an amount of a non-immunogenic compound covalently attached to said cell surface directly or by means of linking moieties, so that recognition of said antigenic determinants on said cell surface is blocked by said attached non-immunogenic compound.

2. The cellular composition of claim 1 wherein said non-immunogenic compound is a polyalkylene glycol.

3. The cellular composition of claim 2 wherein said non-immunogenic compound is methoxypolyethylene glycol.

4. The cellular composition of claim 1 wherein said non-immunogenic compound is dextran.

5. The cellular composition of claim 1 wherein said non-immunogenic compound is Ficoll.

6. The cellular composition of claim 1 wherein said non-immunogenic compound is arabinogalactan.

7. The cellular composition of claims 1, 2, 3, 4, 5 or 6 wherein said linking moieties are covalently attached to said antigenic determinants on said cell surface.

8. A method of producing a non-immunogenic viral cell, said method comprising:

covalently attaching an amount of a non-immunogenic compound to the cell surface, directly or by means of a linking moiety, so that said non-immunogenic compound blocks recognition of antigenic determinants on the cell surface and yields a non-immunogenic cell.

9. The method of claim 8 wherein said linking moiety is covalently attached to said antigenic determinants on said cell surface.

10. The method of claim 8 further comprising transfusing a human with said non-immunogenic viral cell.

11. A non-immunogenic cell produced by the method of claim 8.

12. A method of decreasing phagocytosis of a viral cell, said method comprising:

selecting a viral cell for introduction into a mammal, said viral cell having a cell surface and antigenic determinants on said cell surface;

covalently attaching an amount of a non-immunogenic compound to said cell surface, directly or by means of a linking moiety, so that said non-immunogenic compound blocks recognition of said antigenic determinants on said cell surface and produces a non-immunogenic viral cell; and

introducing said non-immunogenic viral cell into a mammal, wherein phagocytosis of said non-immunogenic viral cell is decreased as compared to phagocytosis of said viral cell.

13. The method of claim 12 wherein said linking moiety is covalently attached to said antigenic determinant on said cell surface.

14. A method of decreasing an adverse reaction to a introduction of a viral particle into a mammalian body, said method comprising:

selecting a viral particle for introduction into a mammal, said viral particle having a surface;

covalently attaching an amount of a non-immunogenic compound to said surface, directly or by means of a linking moiety, so that said non-immunogenic compound blocks recognition of said viral particle and produces a non-immunogenic viral particle; and

introducing into a mammal said non-immunogenic viral particle, wherein adverse reaction to said introduction of said non-immunogenic viral particle is decreased as compared to transfusion of said selected viral particle.

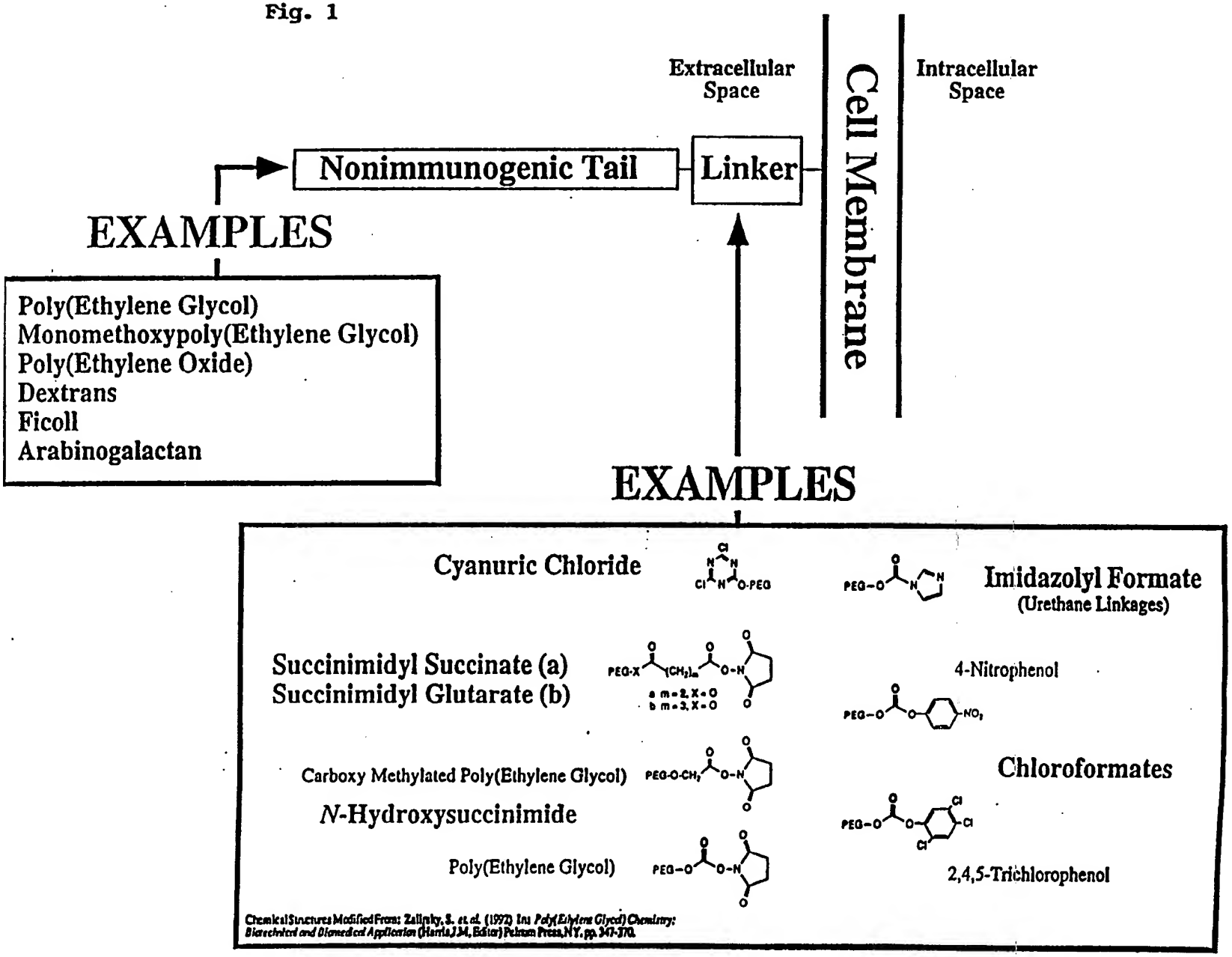
15. The method of claim 14 wherein said mammal is a human.

16. A method of decreasing antibody-induced aggregation of viral particles, said method comprising:

covalently attaching an amount of a non-immunogenic compound to a surface of said particles, directly or by means of linking moieties, so that said non-immunogenic compound blocks recognition of antigenic determinants on the particle surface, so as to produce non-aggregating particles, wherein antibody-induced aggregation of said non-aggregating particles is decreased as compared to antibody-induced aggregation of said particles prior to attachment of the compound.

17. The method of claim 16 wherein said linking moieties are covalently attached to said antigenic determinants on said cell surface.

Fig. 1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Cell Membrane
Intracellular Space
Nonimmunogenic Tail
Linker
EXAMPLES
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Monomethoxypoly(Ethylene Glycol)
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Dextrans
Ficoll
Arabinogalactan
EXAMPLES
Cyanuric Chloride
Imidazolyl Formate
(Urethane Linkages)
Succinimidyl Succinate (a)
Succinimidyl Glutarate (b)
4-Nitrophenol
Carboxy Methylated Poly(Ethylene Glycol)
N-Hydroxysuccinimide
Chloroformates
Poly(Ethylene Glycol)
2,4,5-Trichlorophenol
Chemical Structures Modified From: Zalipsky, S. et al. (1992) In: Poly(Ethylene Glycol) Chemistry: Biotechnical and Biomedical Application (Harris J.M, Editor) Plenum Press, NY. pp. 347-370.

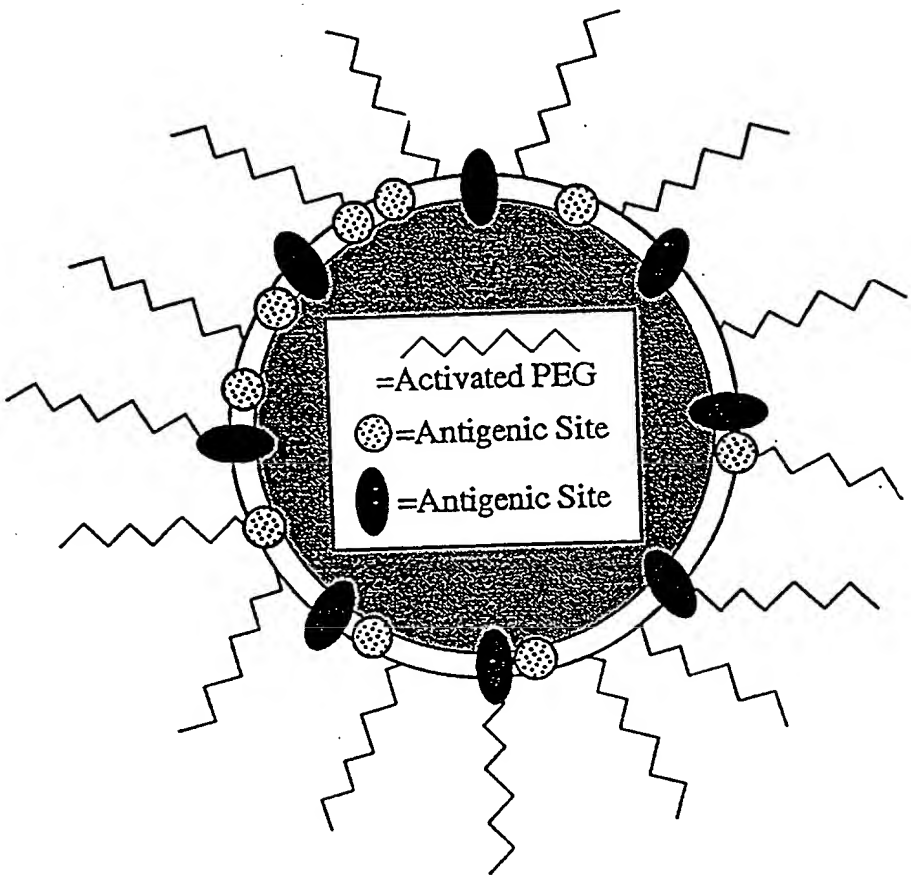


FIG. 2

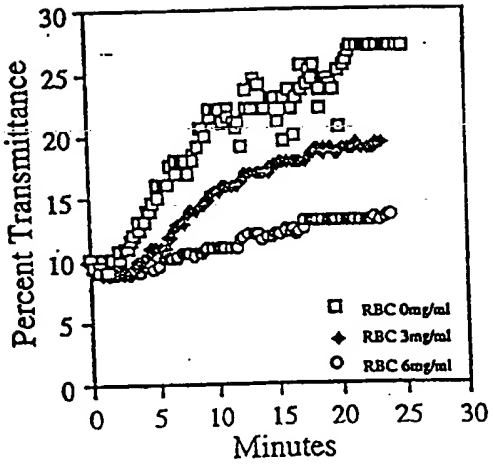
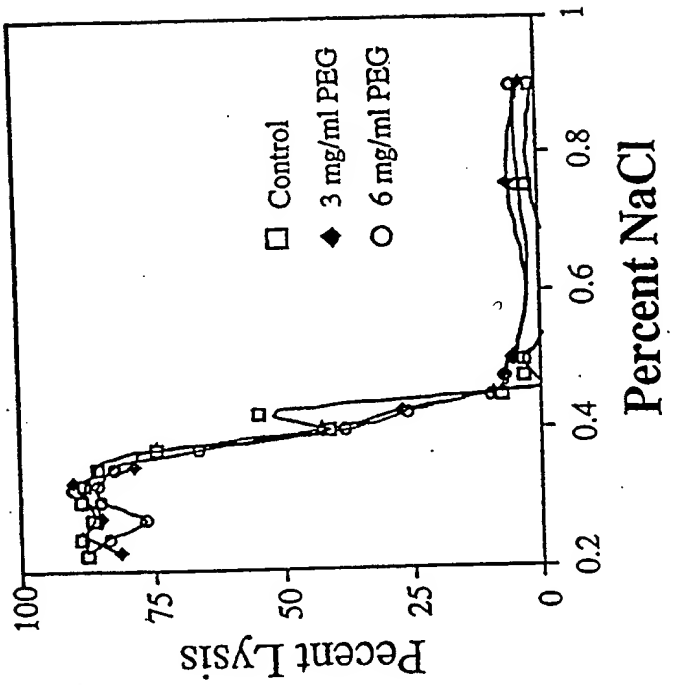

Percent Transmittance
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Minutes
RBC 0mg/ml
RBC 3mg/ml
RBC 6mg/ml

Fig. 3


Percent Lysis
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75
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0.4
0.6
0.8
1
Percent NaCl
Control
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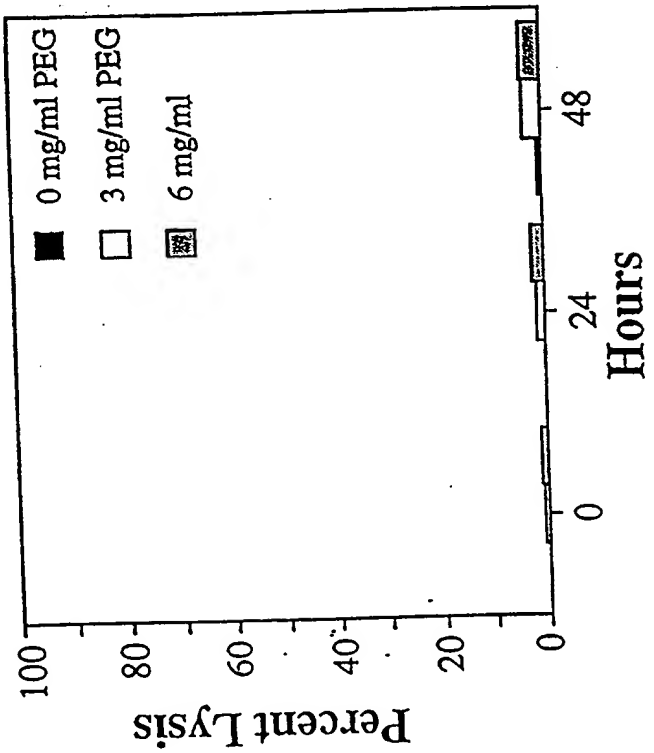
6 mg/ml PEG

Fig. 5


Percent Lysis
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80
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40
20
0
0
24
48
Hours
0 mg/ml PEG
3 mg/ml PEG
6 mg/ml

Fig. 4

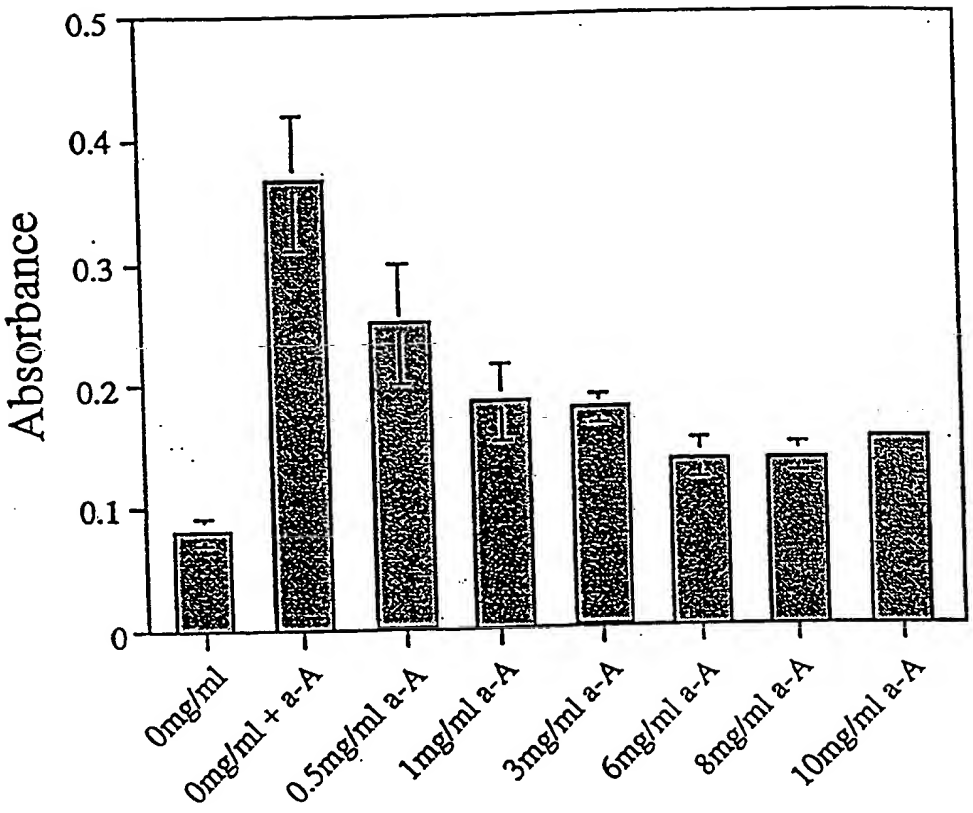

Absorbance
0.5
0.4
0.3
0.2
0.1
0
0mg/ml
0mg/ml + a-A
0.5mg/ml a-A
1mg/ml a-A
3mg/ml a-A
6mg/ml a-A
8mg/ml a-A
10mg/ml a-A

Fig. 6

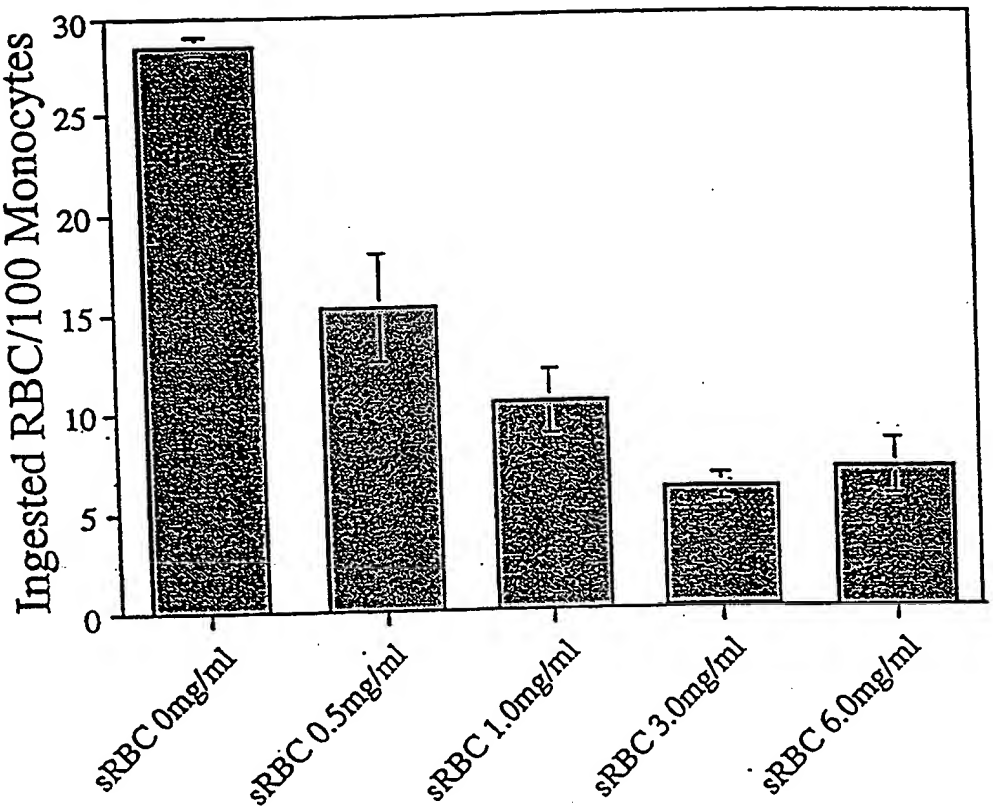

Ingested RBC/100 Monocytes
30
25
20
15
10
5
0
sRBC 0mg/ml
sRBC 0.5mg/ml
sRBC 1.0mg/ml
sRBC 3.0mg/ml
sRBC 6.0mg/ml

Fig. 7

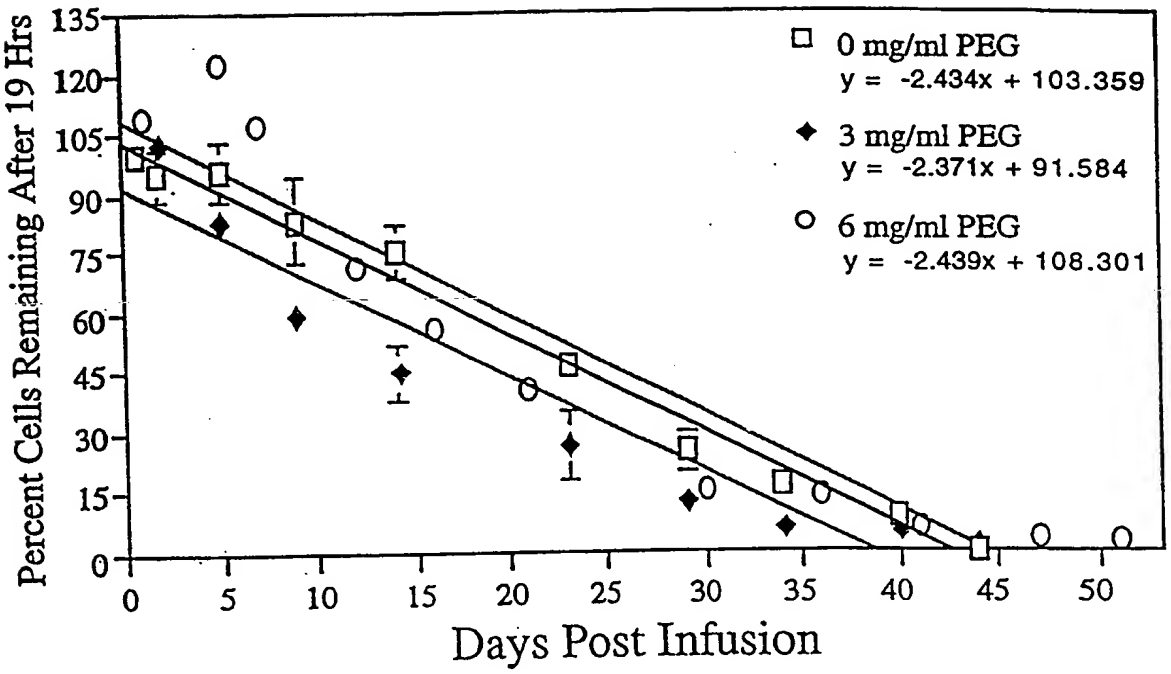

Percent Cells Remaining After 19 Hrs
135
120
105
90
75
60
45
30
15
0
0
5
10
15
20
25
30
35
40
45
50
Days Post Infusion
0 mg/ml PEG
y = -2.434x + 103.359
3 mg/ml PEG
y = -2.371x + 91.584
6 mg/ml PEG
y = -2.439x + 108.301

Fig. 8

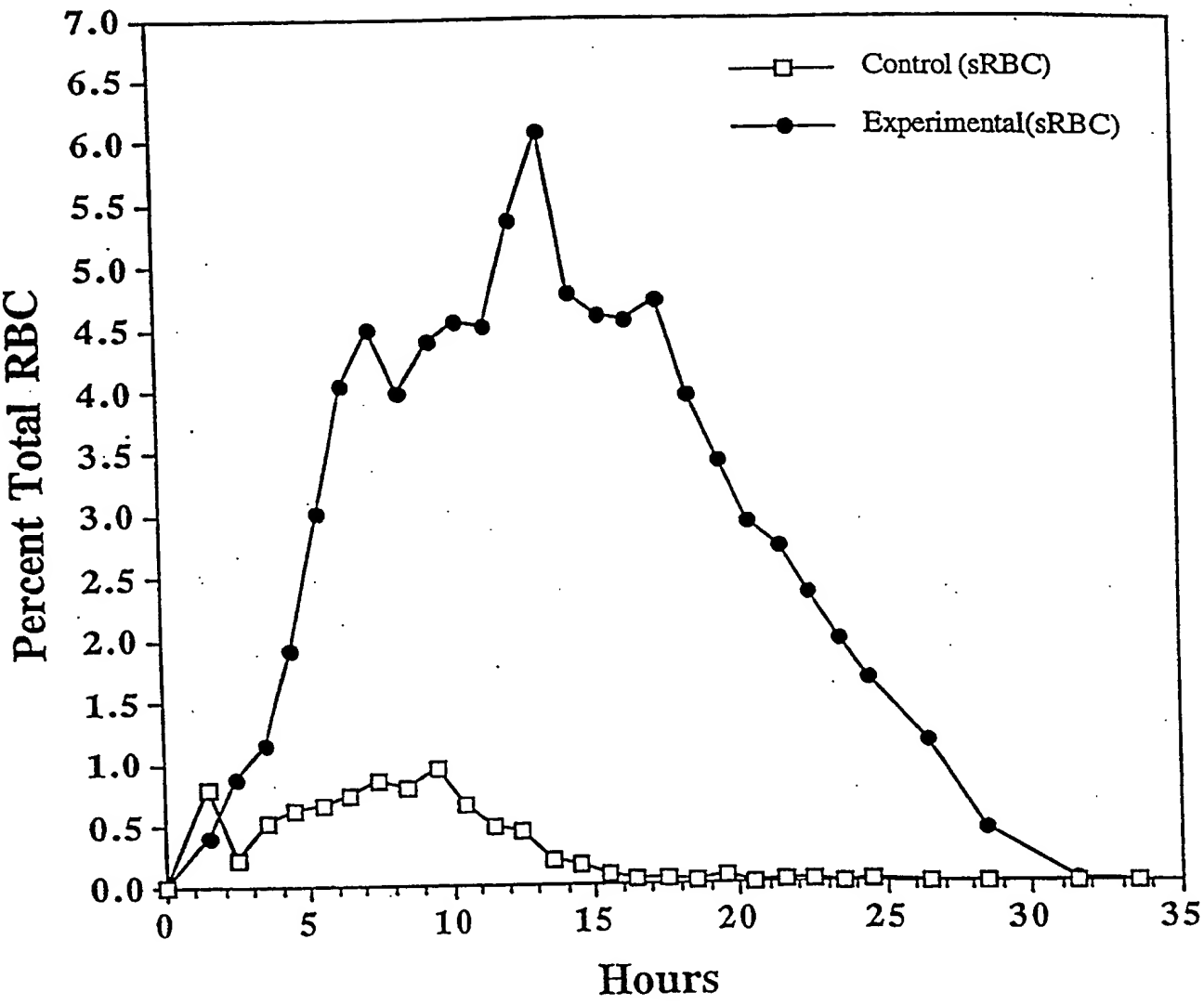
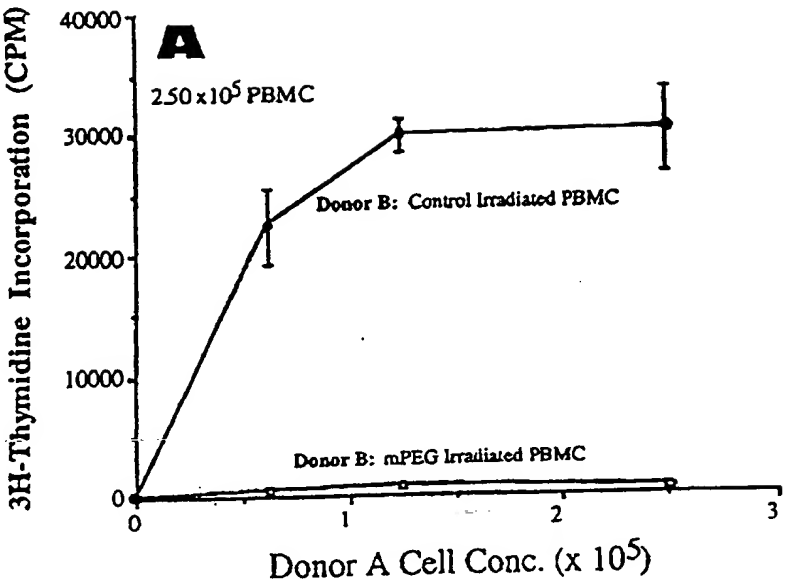

Control (sRBC)
Experimental(sRBC)
Percent Total RBC
Hours
7.0
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
5
10
15
20
25
30
35

Fig. 9

FIGURE 10


A
2.50 x10^5 PBMC
3H-Thymidine Incorporation (CPM)
40000
30000
20000
10000
0
Donor B: Control Irradiated PBMC
Donor B: mPEG Irradiated PBMC
0
1
2
3
Donor A Cell Conc. (x 10^5)

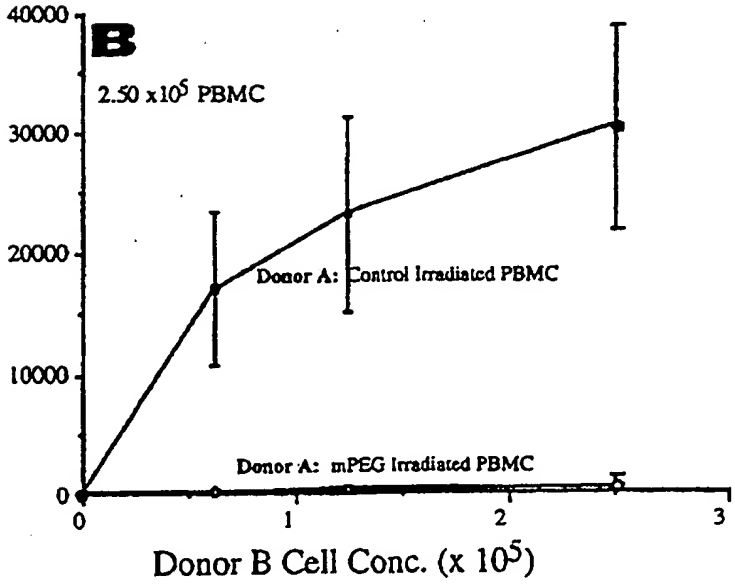

B
2.50 x10^5 PBMC
40000
30000
20000
10000
0
Donor A: Control Irradiated PBMC
Donor A: mPEG Irradiated PBMC
0
1
2
3
Donor B Cell Conc. (x 10^5)

FIGURE 11

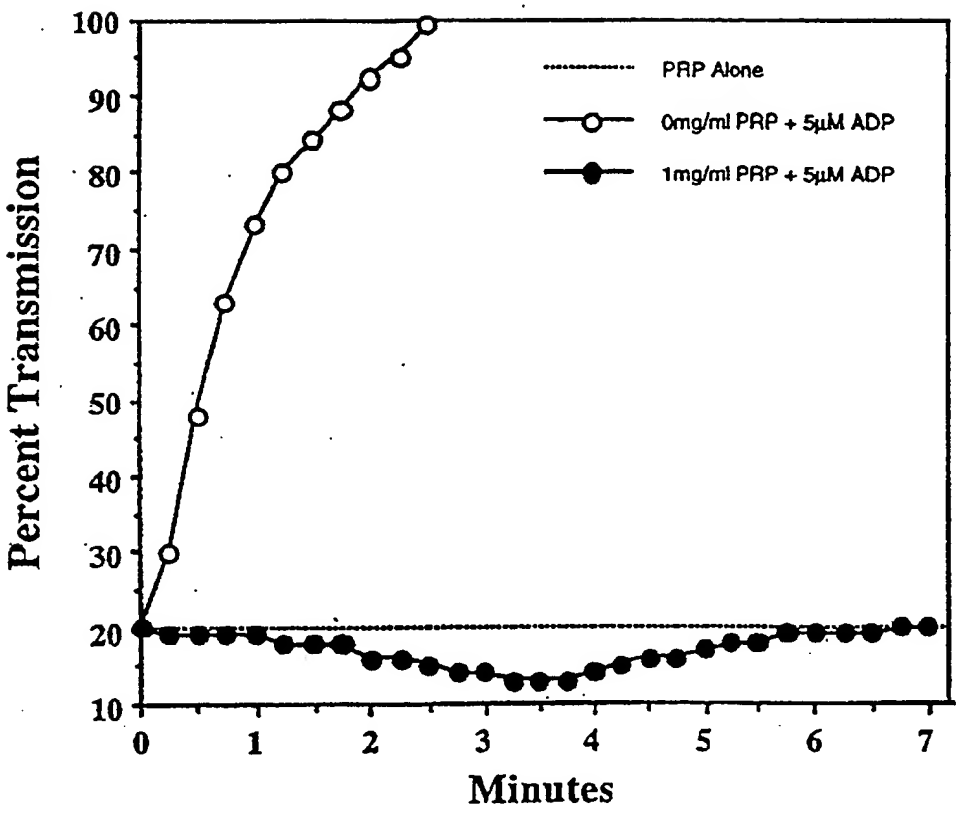
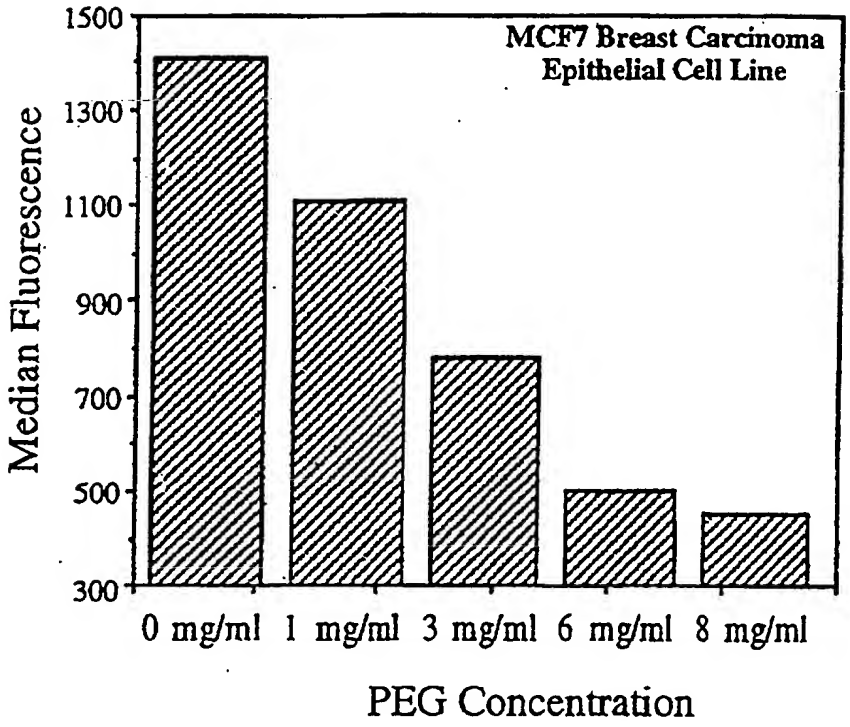

Percent Transmission
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
Minutes
PRP Alone
0mg/ml PRP + 5µM ADP
1mg/ml PRP + 5µM ADP

FIGURE 12


MCF7 Breast Carcinoma
Epithelial Cell Line
Median Fluorescence
1500
1300
1100
900
700
500
300
0 mg/ml
1 mg/ml
3 mg/ml
6 mg/ml
8 mg/ml
PEG Concentration

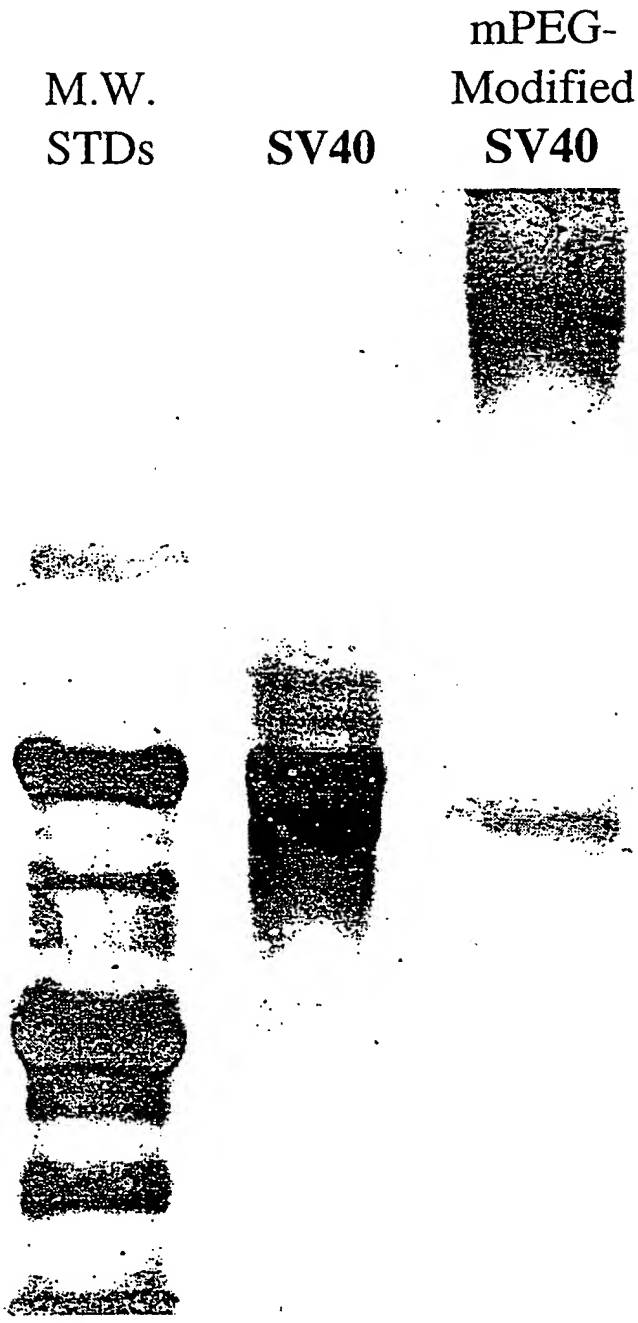


FIG. 13

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 98/13198

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K47/48 C12N15/88 C12N15/86

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 21036 A (VIAGENE INC) 11 July 1996 see page 2, line 30-34 see page 10, line 30 - page 11, line 3 see page 12, line 7-14 see page 14, line 1-26 see claims 15-17 ---	1-4,8, 10-12, 14-16
X	WO 96 41606 A (THEREXSYS LTD) 27 December 1996 see page 4, line 11-27 see page 5, line 3-4; claims 1,2,15,16,23; figure 1; example 11 --- -/--	1-3,8, 10-12, 14-16

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
24 September 1998	08/10/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Veronese, A

INTERNATIONAL SEARCH REPORT

Inter ınal Application No
PCT/US 98/13198

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
E	WO 98 32466 A (FRANCIS GILLIAN ELIZABETH ;FISHER DEREK (GB); MALIK FAROOQ (GB); P) 30 July 1998 see page 1, line 1-12 see page 16, line 23 see claims 1,2,7	1-3,8
Y	JEONG S T ET AL: "DECREASED AGGLUTINABILITY OF METHOXY-POLYETHYLENE GLYCOL ATTACHED RED BLOOD CELLS: SIGNIFICANCE AS A BLOOD SUBSTITUTE" ARTIFICIAL CELLS, BLOOD SUBSTITUTES, AND IMMOBILIZATION BIOTECHNOLOGY, vol. 24, no. 5, 1996, pages 503-511, XP000673685 see the whole document	1-4,6-17
P,Y	WO 97 28254 A (BIOMED FRONTIERS INC ;SEABORN GEORGE STEPHEN (GB); BYUN SI MYUNG ( ) 7 August 1997 see the whole document	1-4,6-17
Y	AL FASBENER ET AL.: "Complexes of Adenovirus with polycationic polymers and cationic Lipids Increase the efficiency of Gene Transfer in Vitro and in Vivo" THE JOURNAL OF BIOLOGICAL CHEMISTRY, 7 March 1997, XP002078069 see page 6482 - page 6483; figures 4,5	1-4,6-17
T	M.CHILLON: "Adenovirus complexed with polyethylene glycol and cationic lipid is shielded from neutralizing antibodies in vitro." GENE THERAPY,July 1998, XP002078070 see the whole document	1-17

INTERNATIONAL SEARCH REPORT

I. ..rnational application No.
PCT/US 98/ 13198

Box I Obs rvations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim(s) 10- 12-15
is(are) directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompani d by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter inal Application No
PCT/US 98/13198

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9621036 A	11-07-1996	AU 4690596 A	24-07-1996
WO 9641606 A	27-12-1996	AU 6011496 A	09-01-1997
		EP 0831922 A	01-04-1998
		AU 1185097 A	14-07-1997
		WO 9722363 A	26-06-1997
WO 9832466 A	30-07-1998	NONE	
WO 9728254 A	07-08-1997	AU 1555297 A	22-08-1997